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Dijkman, Willem P.; de Gonzalo, Gonzalo; Mattevi, Andrea; Fraaije, Marco W.

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MINI-REVIEW

Flavoprotein oxidases: classification and applications

Willem P. Dijkman · Gonzalo de Gonzalo · Andrea Mattevi · Marco W. Fraaije

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Abstract This review provides an overview of oxidases that utilise a flavin cofactor for catalysis. This class of oxidative flavoenzymes has shown to harbour a large number of biotechnologically interesting enzymes. Applications range from their use as biocatalysts for the synthesis of pharmaceutical compounds to the integration in biosensors. Through the recent developments in genome sequencing, the number of newly discovered oxidases is steadily growing. Recent progress in the field of flavoprotein oxidase discovery and the obtained biochemical knowledge on these enzymes are reviewed. Except for a structure-based classification of known flavoprotein oxidases, also their potential in recent biotechnological applications is discussed.

Keywords Flavoproteins · Oxidases · Biocatalysis · Biosensors · Flavin · Oxygen

Introduction

Selective oxidations are often key in synthetic routes towards valuable chemicals. Chemical oxidation methods typically require harsh conditions and polluting reagents and often suffer from poor chemoselectivity and enantioselectivity. Nature, on the other hand, has come up with a versatile set of enzymes performing a wide variety of selective oxidation reactions. Known oxidative enzyme classes are the oxidases, oxygenases and dehydrogenases. Oxygenases require reducing equivalents [e.g. NAD(P)H] and molecular oxygen for activity, while dehydrogenases utilise organic coenzymes (for example pyrroloquinoline quinone, quinones or NAD^+) as electron acceptors. The coenzyme dependence of these enzymes can compromise cost-effective biotechnological applications. Instead of (often expensive) coenzymes, oxidases merely require molecular oxygen as oxidant (electron acceptor) for catalysis. This feature makes them valuable enzymes for industrial applications that range from oxidase-based biosensors to their application as biocatalysts in the synthesis of valuable chemicals. For an organism, however, oxidases are much less favourable. By the direct reduction of molecular oxygen, electrons are lost and cannot be used in subsequent metabolic events, as in the case of dehydrogenases. Another physiological relevant issue concerning oxidases is their inherent reactivity to produce reduced dioxygen species. While some oxidases are capable to reduce dioxygen to harmless water, most oxidases generate instead hydrogen peroxide as by-product, and even in some cases, the even more toxic superoxide O_2^- is formed. The features above provide logic for why oxidases are relatively rare enzymes.

Most oxidases rely on a tightly bound cofactor for their activity, and only a few examples of cofactor independent oxidases have been described (Fetzner and Steiner 2010). This is due to the fact that amino acids are very poor in mediating redox reactions. To equip enzymes with oxidising power, nature has evolved several different redox cofactors. For oxidases, there is a bias towards two types of cofactors resulting in two main oxidase families. One family utilises copper in mono- and trinuclear centers or a copper atom combined with a quinone cofactor. The reader is refereed to many reviews on this topic (Guengerich 2012; Ridge et al. 2008). This review will focus on the other major oxidase family: the flavin-containing oxidases.

W. P. Dijkman · G. de Gonzalo · M. W. Fraaije (✉)
Molecular Enzymology Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen,
Nijenborgh 4,
9747 AG Groningen, The Netherlands
e-mail: m.w.fraaije@rug.nl
URL: www.rug.nl/staff/m.w.fraaije/research

A. Mattevi
Department of Biology and Biotechnology,
University of Pavia, Via Ferrata 1,
27100 Pavia, Italy

Flavin cofactors can be present as flavin adenine dinucleotide (FAD) or, less often, as flavin mononucleotide (FMN) (Macheroux et al. 2011). In most flavoprotein oxidases, the flavin is the sole cofactor, but in some oxidases, either FAD or FMN works in concert with another cofactor. The catalytic cycle of flavoprotein oxidases consists of two half reactions. In the reductive half reaction (step 1, Fig. 1), the organic substrate is oxidised by a two-electron transfer, which results in a fully reduced flavin (hydroquinone), and the oxidised product or product intermediate. For this half reaction, and analogous to nicotinamide-catalysed oxidations, a proper positioning of the substrate with respect to the reactive N5 atom of the flavin cofactor is required (Fraaije and Mattevi 2000). Regeneration of the oxidised cofactor by dioxygen takes places in the subsequent oxidative half reaction, which is a far-from-trivial reaction (steps 2 and 3, Fig. 1). In fact, the ability to use molecular oxygen as electron acceptor sets flavoprotein oxidases apart from all other flavoproteins. The reaction is spin forbidden because the electrons in dioxygen are in a triplet state. This restriction is overcome by a stepwise electron transfer (Mattevi 2006). The redox potential of the oxidised flavin–hydroquinone flavin couple varies between −400 and +120 mV in a protein environment, which is much lower than the potential of the dioxygen–hydrogen peroxide couple. As a result, flavin-mediated oxidations that involve dioxygen as electron acceptor are thermodynamically favourable. Yet, the protein microenvironment around the flavin cofactor determines how efficiently dioxygen can be utilised as electron acceptor (Chaiyen et al. 2012).

The success of flavin-containing oxidases in biotechnological applications is for a large part due to their interesting catalytic properties, as they can perform a wide variety of different oxidation reactions with exquisite chemoselectivity, regio- and/or enantioselectivity while merely using molecular oxygen as oxidant. The best-known flavoprotein oxidase is probably glucose oxidase, which has been produced and applied already for several decades (Bankar et al. 2009). Other known examples of applied oxidases are D-amino acid oxidase (Pollegioni et al. 2008), employed in synthesis of antibiotics, and monoamine oxidases, used for the preparation of enantiopure fine chemicals (Turner 2011). However, in the last decade, a large number of flavoprotein oxidases with other substrate scopes and reactivities have been discovered (Winter and Fraaije 2012). From detailed biochemical studies on these novel oxidases, new insights have been obtained on the catalytic properties of these enzymes. Besides oxidases that are able to catalyse relatively simple oxidations (for example the oxidation of alcohols to aldehydes or ketones), also more complex oxidative reactions can be catalysed [for example oxidative C–C bond formation by reticuline oxidase (Winkler et al. 2008)]. The architecture of the active site of each oxidase clearly determines its substrate acceptance profile and oxidation reactivity. Moreover, the active site also contains the structural requirements to allow oxygen to reach the flavin and to facilitate dioxygen reduction by the reduced cofactor (Baron et al. 2009). In some cases, the active site entails even an additional catalytic property: It catalyses the formation of a covalent flavin-protein bond. By this, the flavin cofactor is covalently tethered to the protein, preventing dissociation and increasing the redox potential (Fraaije et al. 1999)

Comparison of the currently available flavoprotein oxidase sequences and structures reveals that the oxidases belong to several structurally distinct flavoprotein families. In this review, we provide an inventory of currently known

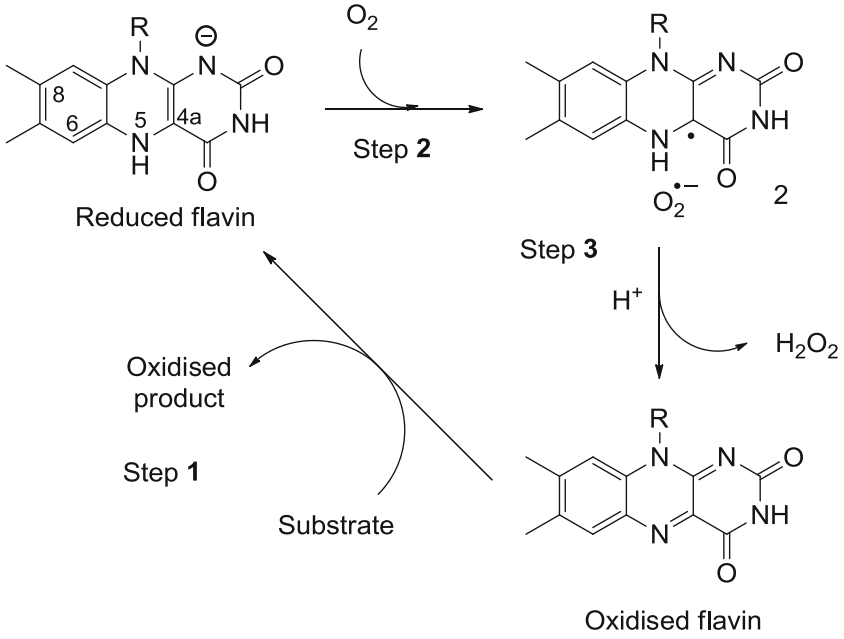

Fig. 1 Catalytic cycle of the flavin cofactor in flavoprotein oxidases

flavoprotein oxidases. Based on sequence homology and the available structural information, a comprehensive classification is proposed (Table 1). The distinct flavoprotein oxidase families will be described in more detail in the next paragraphs, while the last paragraphs will describe several recent advances on applications of flavoprotein oxidases.

The GMC-type oxidase family

The glucose–methanol–choline oxidoreductase (GMC) flavoprotein family contains several well-known oxidases. The protein sequence of each member of this family entails a conserved N-terminal FAD binding domain, called GMC_oxred_N (Pfam00732) in the Pfam database (Finn et al. 2010). This domain includes the typical GxGxxG/A sequence motif, which is indicative for the Rossmann fold that is involved in binding the ADP moiety of the FAD. Less conserved is the C-terminal region, which forms the substrate binding domain (Kiess et al. 1998). It contains only one generally conserved residue, an active-site histidine, which can assist in substrate oxidation and FAD reoxidation by molecular oxygen. Mutagenesis studies on glucose oxidase has revealed that protonation of this histidine (His516 in GO) is essential for catalysing dioxygen reduction (Roth and Klinman 2003). Nevertheless, exceptions to this rule have already been discovered. For instance, in choline oxidase, the function of the histidine has been taken over by the positively charged amine moiety of the choline substrate molecule (Gadda 2012).

Known GMC-type oxidases act on primary and secondary alcohols, forming the corresponding aldehydes or ketones. Examples are glucose oxidase (EC 1.1.3.4; Fig. 2a), cholesterol oxidase (EC 1.1.3.6), pyranose oxidase (EC 1.1.3.10), methanol oxidase (EC 1.1.3.13) and aryl alcohol oxidase (EC 1.1.3.7). Choline oxidase (EC 1.1.3.17) is a notable exception as it is able to perform two sequential oxidations on choline, yielding the carboxylic acid, trimethylglycine, as shown in Fig. 3 (Fan et al. 2004).

Several oxidases were found to contain a covalently linked FAD cofactor (choline oxidase and pyranose oxidase), where the flavin is attached via an 8α-N^3-histidyl bond. Nevertheless, the majority of GMC-type oxidases contain a dissociable FAD cofactor that is tightly bound to the protein. Mechanistic details have been established for a number of GMC-type oxidases, including glucose oxidase, pyranose oxidase, aryl alcohol oxidase and choline oxidase. The mechanism by which GMC-type oxidases oxidise their substrates involves a direct hydride transfer from the substrate to the N5 atom of the FAD cofactor. This reaction is facilitated by proton abstraction, typically promoted by a histidine acting as a general base.

In addition to oxidases, the GMC flavoprotein family also contains other types of flavoenzymes. A number of flavoprotein dehydrogenases (for example glucose dehydrogenase and cellobiose dehydrogenase) and even non-redox flavoproteins (for example hydroxynitrile lyase) are members of this superfamily. In contrast to the sequence-related oxidases, these enzymes are ineffective in utilising molecular oxygen as electron acceptor. At present, there is no sequence motif known that can discriminate between GMC-type oxidases and other GMC-type enzymes. The same holds true for most of the flavoprotein oxidase families below. More knowledge on the precise mechanism by which flavoproteins can tune their oxygen reactivity may change this situation and would perhaps enable more reliable annotation of flavoprotein-encoding genes into oxidases, facilitating effective genome mining for novel flavoprotein oxidases.

The VAO-type oxidase family

The second flavoprotein family, which includes oxidases, is the vanillyl alcohol oxidase (VAO) flavoprotein family, which was named after a fungal oxidase, VAO (EC 1.1.3.38; Fraaije et al. 1998; Leferink et al. 2008; Mattevi

Table 1 Classification of the major flavoprotein oxidase families

Flavoprotein oxidase family	GMC	VAO	AO	SO	ACO	HAO
Cofactor type	FAD	FAD	FAD	FAD	FAD	FMN
Cofactor binding mode:						
Noncovalent	+	+	+	+	+	+
Covalent	+	+	+	−	−	−
Typical Substrates	Alcohols	Alcohols Amines	Amines	Thiols	Acyl CoA	Hydroxyacids
Oxidase prototype (PDB)	Glucose oxidase (1CF3)	Vanillyl alcohol oxidase (1VAO)	D-Amino acid oxidase (1KIF)	Erv2p sulfhydryl Oxidase (1JRA)	Acyl CoA oxidase (1IS2)	Glycolate oxidase (1GOX)

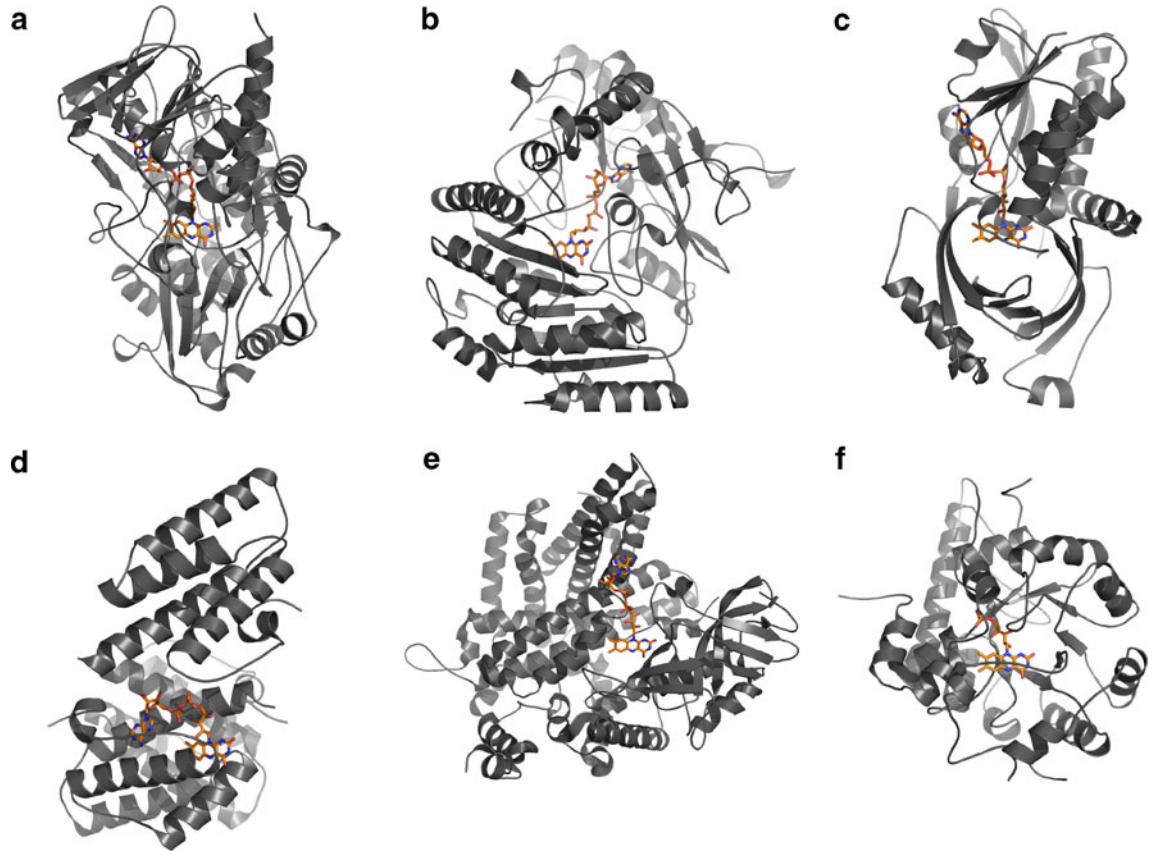

Fig. 2 Structures of representative members of the different flavin-dependent oxidases with the FAD or FMN cofactor highlighted in *gold*. **a** Glucose oxidase (pdb 1CF3). **b** Vanillyl alcohol oxidase (pdb 1VAO). **c** D-amino acid oxidase (pdb 1KIF). **d** Sulfhydryl oxidase Erv2p (pdb 1JRA). **e** acyl CoA oxidase (pdb 1FS2). **f** Glycolate oxidase (pdb 1GOX)

et al. 1997). Similar to GMC-type flavoproteins, all VAO-type flavoproteins contain a distinct FAD binding domain (FAD_binding_4 domain, Pfam01565) in the N-terminal half of the protein (Fig. 2b). This domain includes the so-called PP loop where the pyrophosphate moiety of the FAD interacts with the protein (Mattevi et al. 1997). The vanillyl alcohol oxidase crystal structure revealed for the first time how a FAD cofactor is covalently tethered to a protein via a histidine residue. Indeed, the VAO flavoprotein family is relatively rich in covalent flavoproteins (estimated 25 % contain a histidyl-FAD). It has been established that, by covalent attachment of the FAD cofactor to a histidine, the redox potential of the cofactor is increased to a large extend

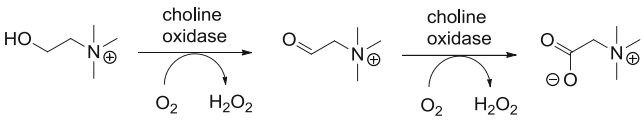

Fig. 3 Choline oxidase-catalysed sequential oxidation of choline into trimethylglycine

(Fraaije et al. 1999). As a result, the number of feasible electron acceptors is limited, leaving molecular oxygen as one of the few candidates. This explains why many VAO flavoproteins act as oxidases. Recently, it has been discovered that several VAO-type oxidases even contain a bicovalently linked FAD cofactor. In these cases, in addition to the histidyl bond, the FAD is also tethered at the 6 position to a cysteine (Heuts et al. 2009). Except for an effect on the redox potential, it is hypothesised that the second covalent bond allows the protein to create a very open active site, while retaining the FAD cofactor in position for catalysis through the bicovalent anchoring. This is in line with the observation that the described bicovalent flavoprotein oxidases all accept rather bulky substrates (Heuts et al. 2007a).

For VAO-type oxidases, some details on the oxygen reactivity have been elucidated. For the VAO-type cholesterol oxidase and alditol oxidase (EC 1.1.3.41) specific oxygen channels have been described, guiding dioxygen to the FAD (Baron et al. 2009; Coulombe et al. 2001). In alditol oxidase, all proposed channels are situated in the C-terminal part of the protein and lead to one oxygen entry site

in the active site. Based on this finding, a mutant of an L-galactonolactone dehydrogenase (EC 1.3.2.3) has been engineered in which such an oxygen entry in the active site was created. The mutation indeed resulted in the creation of an oxidase (Leferink et al. 2009).

In the last decade, a large number of novel VAO-type oxidases have been discovered and studied (Leferink et al. 2008; Winter and Fraaije 2012). In addition to alcohol oxidations, leading in most cases to aldehydes or ketones, many other bonds can be oxidised. Vanillyl alcohol oxidase itself was shown to perform alcohol and amine oxidations, hydroxylations of alkyl-phenols and ether bond cleavage (Fraaije et al. 1995). Another remarkable example of a VAO-type oxidase is reticuline oxidase, also known as berberine bridge enzyme (EC 1.21.3.3). This enzyme is able to form a C–C bond, creating the intramolecular bond between a methyl group and an aromatic carbon in the conversion from (*S*)-reticuline to (*S*)-scoulerine, a plant alkaloid (Winkler et al. 2008). Elucidation of the crystal structure of this plant enzyme has revealed that the reaction is triggered by hydride transfer from the substrate to the flavin cofactor. VAO-type oxidases that can catalyse an oxidative C–C bond formation are not restricted to plants. Prosolanapyrone-II oxidase (EC1.1.3.42) from the fungus *Alternariasolani* performs an intramolecular Diels–Alder reaction, a [4+2] cycloaddition in which a conjugated diene reacts with a carbon–carbon double bond (Kasahara et al. 2010). In addition, other microbial C–C and C–O forming VAO-type oxidases have been described in recent literature (Winter and Fraaije 2012).

The amine oxidase family

The amine oxidase (AO)-type oxidases belong to another distinct structural FAD-containing flavoprotein family. As the name suggests, most enzymes are solely active on amines. Prototype oxidases for this family of flavoprotein oxidases are microbial amino acid oxidases (Fig. 2c) and eukaryotic monoamine oxidases. Beside C–N oxidising oxidases, a few alcohol and thioether oxidases have been found that belong to this family. This shows that AO-type oxidases cover a wide range of different reactions. The individual enzymes, however, can in most cases perform only one type of reaction.

All members of this family (Pfam01593 and Pfam01266) share a similar domain in the N-terminal half of the protein, responsible for FAD binding. Covalent binding through a histidyl or cysteinyl bond on the 8α position of FAD is frequently observed. Most AO-type oxidases act on a carbon–nitrogen bond of a primary or secondary amine. The catalytic cycle of these oxidases starts with proton abstraction from the amine or ammonium group by an active side base like histidine (Trickey et al. 1999) or a catalytic dyad of histidine and tyrosine (Leys et al. 2003). Hydride transfer to the enzyme-bound FAD results in the formation of an imine product intermediate, which can spontaneously hydrolyse to an aldehyde and amine (Fitzpatrick 2010; Trickey et al. 1999).

Among the known AO-type oxidases, also thioether oxidases have been identified: prenylcysteine lyase (EC 1.8.3.5) and farnesylcysteine lyase (EC 1.8.3.6). The proposed catalytic mechanism for prenylcysteine oxidase is quite distinct from other FAD-catalysed oxidations. The C–S bond is not activated by an active site base; the first step is hydride transfer to FAD. The resulting thiocarbenium is hydrolysed to farnesal, an aldehyde and cysteine (Fig. 4) (Digits et al. 2002). The overall products of the reaction, therefore, resemble the ones obtained upon secondary amine oxidation.

Another unusual AO-type oxidase is nikD, which is involved in the biosynthesis of the antibiotic nikkomycin. This oxidase produces the aromatic compound picolinate by a 4-electron oxidation of Δ-piperideine-2-carboxylate (Fig. 5). In the proposed mechanism, the reaction is initiated with oxidation of a carbon–nitrogen bond. After tautomerisation and isomerisation of the formed imine, the same carbon–nitrogen bond is oxidised again resulting in the final, aromatic product (Bruckner and Jorns 2009).

In contrast to all other members of this flavoprotein oxidase family, including monomeric sarcosine oxidase (EC 1. 5.3.1), heterotetrameric sarcosine oxidase does not rely only on FAD as cofactor. The α-subunit of this tetrameric enzyme binds NAD^+, but has no described catalytic function. The β-subunit resembles monomeric sarcosine oxidase, but contains both FAD and covalently bound FMN. In this oxidase subunit, sarcosine is oxidised to the corresponding imine by FAD. With the help of the γ and δ subunits, heterotetrameric sarcosine oxidase can interact with 5,10-CH_2–H_4 folate synthase, forming a channel, which prevents hydrolysis of the imine. The reaction of the imine product reacts with tetrahydrofolate resulting in the formation of 5,10-CH_2-tetrahydrofolate and glycine (Chen et al. 2006).

The sulfhydryl oxidase family

A number of FAD-containing oxidases acting on thiols have been discovered in the last decade. The most important role of these so-called sulfhydryl oxidases is the formation of disulphide bonds in proteins by oxidising cysteine residues. Crystal structures of several sulfhydryl oxidases have been solved, including human, viral and yeast variants (Fig. 2d; Guo et al. 2012; Gross et al. 2004). Based on this, two subfamilies can be identified: Erv-like (Pfam04777) and Ero-like (Pfam04137) sulfhydryl oxidases. The first subfamily is named after Erv1 (EC 1.8.3.2), a protein that was discovered

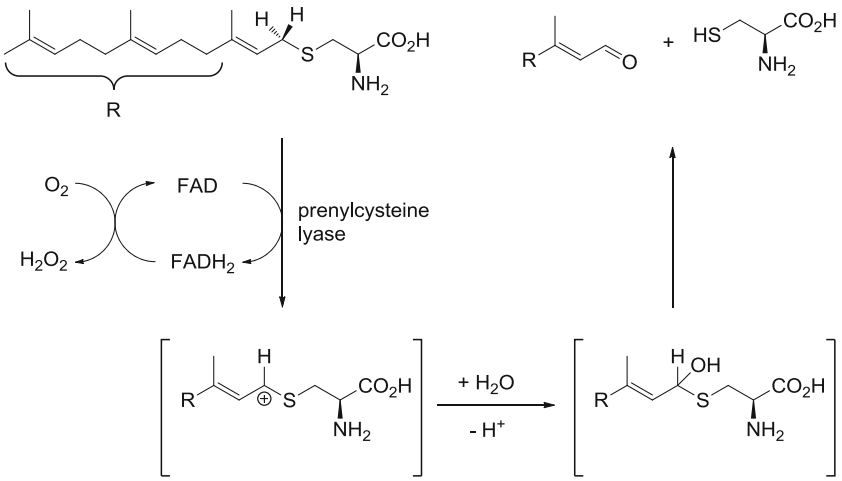

Fig. 4 Proposed mechanism for the formation of farnesal and cysteine as final products in the prenylcysteinelyase-catalysed oxidation of prenylcysteine

in yeast as essential for respiration and viability. The Ero-like subfamily contains homologs of yeast Ero1, which provided insight in the cellular maintenance of a relatively high oxidation potential in the ER compared to the strongly reducing cytosol in which it is embedded (Pollard et al. 1998). Although Ero and Erv oxidases differ in many aspects, including size and substrate spectrum, they belong to the same structural family. Both types of sulfhydryl oxidases have an all-alpha fold, in contrast to all other flavoprotein oxidases. In addition, the atypical FAD binding mode is similar in both subfamilies: the isoalloxazine ring is located between a "barrel" of four helices, with the adenine moiety exposed on the surface. FAD is neither extended (isoalloxazine and adenine far apart) nor bend (both moieties close together): the molecule is bound in an intermediate form (Fass 2008). Covalent binding of the flavin cofactor has not been observed in any member of this flavoprotein oxidase family.

Erv1 from *Saccharomyces cerevisiae* has only 106 residues, which fold around the FAD cofactor, forming five alpha helices (Fass 2008). This is different from most other FAD-containing oxidases, which usually have an N-terminal FAD binding domain and a C-terminal substrate binding domain. The ability of sulfhydryl oxidases to use dioxygen as electron acceptor has been explained by accessibility of the active site. In yeast Ery, a hydrophobic channel is observed leading to the N5-C4a of the FAD, the oxygen-reactive locus of the flavin cofactor (Endo et al. 2010). Rat Erv1 does not have this channel and has poor reactivity towards dioxygen. The inactivity towards dioxygen of some Erv members points in the direction of another physiological terminal electron acceptor. For these enzymes transfer of electrons to cytochrome c oxidase has been proposed,

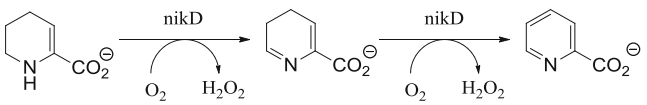

Fig. 5 NikD-catalysed four electron oxidation of Δ-piperideine-2-carboxylate

leading to water instead of hydrogen peroxide, or alternatively, the electrons are shuttled in the respiratory chain instead of direct reduction of dioxygen (Endo et al. 2010).

Sulfhydryl oxidases do not form disulphide bonds directly on target proteins. For Ero, this is achieved via action of protein disulphide isomerase (PDI), the enzyme that introduces disulphide bonds in proteins in the ER (Thorpe et al. 2002). The catalytic disulphide bond in PDI is regenerated by action of Ero1p. This oxidation is not performed directly by the FAD cofactor but rather by a set of conserved cysteines, which form a relay path to transport the electrons to the core of the protein where the isoalloxazine is buried (Araki and Inaba 2012). Erv1 act on target proteins by forming a complex with Mia40 (Guo et al. 2012). Erv1 contains a flexible loop, which functions as electron shuttle, bringing electrons to the active site. In general, as sulfhydryl oxidases do not catalyse direct cysteine oxidations of proteins, their industrial applicability seems to be limited.

The acyl-CoA oxidase-type oxidase family

Acyl-CoA oxidase represents the prototype oxidase of another flavoprotein oxidase family. acyl-CoA oxidase-type (ACO) oxidases contain an N-terminal domain of only α-helices (Pfam02771), a middle domain formed by a β-barrel (Pfam02770), and a C-terminal domain of α-helices (Pfam00441) (Fig. 2e). These oxidases employ FAD as only cofactor, which is located between the middle and the C-terminal α-domain. ACOs (EC 1.3.3.6) catalyse the C_α-C_β oxidation of fatty acids. The C_α proton is abstracted by an active site glutamate to trigger a hydride transfer from the C_β to the N5 of the FAD cofactor (Binzak et al. 1998) As for most other flavoprotein oxidase families, ACO sequence-related acyl-CoA dehydrogenases exist. Whereas the oxidases are predominantly found in peroxisomes, fatty acid degradation in the mitochondria is performed by acyl-CoA dehydrogenases (Tokuoka et al. 2006).

Another recently discovered ACO-type oxidase is the fungal nitroalkane oxidase (EC 1.7.3.1). This FAD-containing oxidase oxidises primary and secondary nitroalkanes to the corresponding aldehydes and ketones with the release of nitrite (Fig. 6). The first step, abstraction of the α-proton from the nitroalkane, is similar to the acyl-CoA oxidase mechanism. The carbanion obtained forms a covalent adduct with the N5 of the flavin, which generates a cationic flavin imine. Attack by water leads to the reduced flavin and the final product, the aldehyde or ketone (Tormos et al. 2010). Reoxidation of the reduced flavin cofactor by molecular oxygen completes the catalytic cycle.

The 2-hydroxyacid oxidase family

A number of flavoprotein oxidases use FMN as prosthetic group. The majority of these oxidases belong to one distinct structural flavoprotein family. Many members of this oxidase family oxidise aromatic or aliphatic 2-hydroxy acids (grouped in EC 1.1.3.5), forming the respective 2-oxoacids (Cunane et al. 2005). Known examples of this oxidase family are glycolate oxidase and L-lactate oxidase. The structure of several members of this family has been solved, providing insights into the fold and the catalytically important residues. The proteins have a β8/α8 TIM barrel structure (Fig. 2f; Umena et al. 2006). A conserved arginine in the active site typically accommodates the carboxyl group of the substrate. The catalytic mechanism of the reductive half reaction for 2-hydroxyacid oxidase (HAO)-type oxidases is still under debate. Studies seem to support both the formation of a carbanion and a hydride transfer mechanism (Furuichi et al. 2008). In both cases, however, a histidine (His265 in lactate oxidase) is involved, abstracting the Cα-proton in the case of the carbanion mechanism and the proton from the Cα-hydroxyl group in the hydride transfer mechanism.

The crystal structure of a FMN-containing nitroalkane oxidase has recently been elucidated (Li et al. 2011). It revealed no structural resemblance with the above-mentioned ACO-type oxidases but rather to the HAO-type flavoprotein oxidase family. This shows that members of the HAO family can perform other reactions than the oxidation of 2-hydroxyacids. The active site of this FMN-containing TIM barrel protein also contains a histidine residue that was found to be crucial for catalysis.

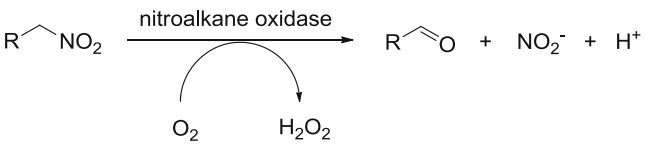

Fig. 6 General scheme of the oxidation of primary nitroalkanes by nitroalkane oxidase in order to obtain aldehydes

Unique flavoprotein oxidases

Several flavoprotein oxidases have been identified for which no or only a few homologs with other activities have been described. These individual oxidases are discussed below. Pyruvate oxidase is an exceptional flavin-containing oxidase as it contains two cofactors, the vitamin B1 and B2 derivatives, thiamine diphosphate (ThDP) and FAD, respectively. The combination of ThDP and FAD in one enzyme is rare, but not restricted to pyruvate oxidase. Acetolactate synthase, also acting on pyruvate, is another example in which both cofactors are bound (Choi et al. 2007). The crystal structure of pyruvate oxidase from *Lactobacillus plantarum* (Muller et al. 1994) has revealed that each monomer of the homotetramer consist of three domains. The FAD cofactor is not buried within the enzyme but is located at the surface between the N-terminal α domain and the middle β domain. ThDP is coordinated by a magnesium ion and located at the C-terminal γ domain. The α and γ domains have a similar topology, whereas the β domain differs (Arjunan et al. 1996). Pyruvate oxidase catalyses the decarboxylation of pyruvate to acetyl phosphate and carbon dioxide, consuming oxygen and yielding hydrogen peroxide. The proposed catalytic mechanism involves both cofactors, ThDP and FAD. The activated C2 carbanion of the thiazolium ring of ThDP attacks pyruvate, leading to decarboxylation. FAD subsequently oxidises the ThDP-substrate adduct. The attack by phosphate releases acetyl phosphate from ThDP, and $FADH_2$ is oxidised by dioxygen to complete the catalytic cycle (Tittmann et al. 2005).

Pyridoxal 5′-phosphate oxidase (PNPO, EC 1.4.3.5) is an essential enzyme in the metabolism of vitamin B6. The enzyme oxidises the amine group of pyridoxamine 5′-phosphate to an aldehyde (Di Salvo et al. 2011), a reaction similar to those catalysed by the AO-type oxidases. The enzyme is also active on pyridoxine 5′-phospate in which the alcohol is oxidised to the aldehyde. In addition, activity on imine and ester moieties has been described (Kazarinoff and McCormick 1973; Kazarinoff and McCormick 1975). PNPO contains a FMN as flavin cofactor and displays no structural resemblance with any of the above discussed flavoprotein oxidases. The crystal structures of human, yeast and two bacterial homologs have been solved. All PNPOs are dimers, with each monomer composed of two domains. The N-terminal domain (Pfam01243) forms a six stranded β-barrel with antiparallel strands. The smaller C-terminal domain (Pfam 10590) adopts a helix–loop–helix conformation. FMN binds in the β-barrel of the protein with the

phosphate moiety near the N-terminus (Biswal et al. 2005). Mechanistic studies have revealed that, as for many other flavoprotein oxidases, oxidation of the substrate is achieved by a direct hydride transfer from the substrate to the N5 of the FMN cofactor (Di Salvo et al. 2011).

Another FAD-containing oxidase that catalyses only one type of reaction is NADPH oxidase (NOX). This enzyme is implicated in a large number of physiological processes in humans. A crucial catalytic property is the ability to generate superoxide as reduced oxygen species. The enzyme is composed of multiple subunits and is membrane bound. Although the crystal structure of NOX has not yet been elucidated, a structure of the subunit $gp91^{phox}$ has been proposed based on sequence alignments and homology models. This shows that the protein contains an N-terminal membrane domain composed of six α-helices. The C-terminal domain is exposed in the cytosol and contains both the NADPH and the FAD binding sites (Debeurme et al. 2010). NOXs have also been described in prokaryotes, being both FAD- and FMN-dependent enzymes depending on the microbial source. These enzymes can be produced as recombinant enzymes, being valuable biocatalysts for regeneration of the $NAD(P)^+$ cofactor.

Xanthine oxidase (XO, EC 1.17.3.2) represents yet another flavin-containing oxidase. The oxidase does not only rely on FAD for activity but also contains two non-identical [2Fe–2S] clusters and a molybdenum cofactor. In fact, the oxidation of the organic substrate occurs at the molybdopterin. The [2Fe–2S] clusters assist in catalysis by facilitating electron transfer from the molybdenum site to the flavin cofactor (Pauff et al. 2007). The FAD cofactor is merely used to transfer the electrons to a suitable electron acceptor. This also explains the atypical oxidations that are catalysed by XO: It can catalyse hydroxylations of a large number of compounds, including pyrimidines, purines and pterins. In contract with typical oxygenases, the inserted oxygen is derived from water. XO is an excellent example of the thin line between oxidases and dehydrogenases. The oxidase can efficiently use dioxygen as electron acceptor, but is unable to reduce NAD^+. This is in sharp contrast with xanthine dehydrogenase (XDH) for which the opposite is true. Yet, both XO and XDH are essentially the same protein (Nishino et al. 2008). XDH can reversibly be interconverted to XO when two disulphide bonds are formed (Nishino et al. 2005). The disulphide bond formation causes a conformational change which disrupts the cavity needed for NAD^+ to reach FAD. Irreversible formation of XO from XDH happens upon proteolytic cleavage of the linker between the FAD and the molybdenum binding domain, again changing the environment of the NAD^+ binding site, leaving the protein only active towards dioxygen. Another special feature of XO is that, depending on the conditions, it produces hydrogen peroxide or superoxide as reduced dioxygen species.

Flavoprotein oxidases in biotechnology

Flavoprotein oxidases have been widely employed in biocatalytic processes. In most cases, the targeted reactions involved the selective oxidation of alcohols or amines. In the last decade, amine-oxidising enzymes have become very popular as biocatalysts. Both monoamine oxidases and amino acid oxidases have been extensively applied in the selective oxidation of amines and amino acids under mild reaction conditions. For instance, a D-amino acid oxidase from yeast has been employed in the industrial preparation of 7-aminocephalosporanic acid (Pillone and Pollegioni 2002), and an engineered bacterial glycine oxidase could be engineered to convert *N*-(phosphonomethyl)glycine (glyphosate; Pedotti et al. 2009). Another development is the use of monoamine oxidases and amino acid oxidases in chemo-enzymatic deracemisation of racemic amines. This concept relies on the ability of the oxidases to oxidise only one enantiomer into the corresponding imine or imino acid, which is chemically (nonselectively) reduced back into the amine. By performing this cycle of enzymatic oxidation and chemical reduction in one pot (cyclic deracemisation), a 100% theoretical yield of an optically pure amine is feasible. For examples of this elegant approach for deracemisation of amines, the reader is referred to a recent review of Turner (2011). Amine-oxidising oxidases have also been combined with other enzymes for the preparation of enantiopure amines (Patel 2011). For example, in 2011, enantiopure (*S*)-amino-3-[3-{6-(2methylphenyl)}pyridyl]-propionic acid was synthesised in 72% yield from the racemic amino acid by combining the (*R*)-amino acid oxidase from *Trigonopsis variabilis* with (*S*)-aminotransferase from *Sporosarcina urea* (Patel et al. 2007). The racemic amino acid mixture was converted by the oxidase into the (*S*)-enantiomer and a keto acid, of which the latter was converted by the aminotransferase into the (*S*)-amino acid. An (*S*)-aminotransferase from *Burkholderia* sp. was also employed with a global yield of 85% (Fig. 7). The process was scaled up to 100 L. An (*S*)-amino acid oxidase has been used for production of 2-amino-3(7-methyl-1*H*-indazol-5-yl)-propionic acid, a key intermediate for the preparation of anti-migraine drugs. Enantiopure (*R*)-amino acid was prepared with 79% global yield by the selective oxidation with the (*S*)-amino acid oxidase from *Proteus mirabilis* followed by the transamination of the keto acid intermediate by a commercially available (*R*)-transaminase (Hanson et al. 2008).

One recent example of a flavoprotein oxidase that can be used for synthetic chemistry is the plant flavoprotein oxidase, reticuline oxidase. This VAO-type oxidase enzyme catalyses the biotransformation of (*S*)-reticuline into (*S*)-scoulerine through the formation of an intramolecular C–C bond at the expense of molecular oxygen. Scoulerine and related compounds often exhibit interesting biological

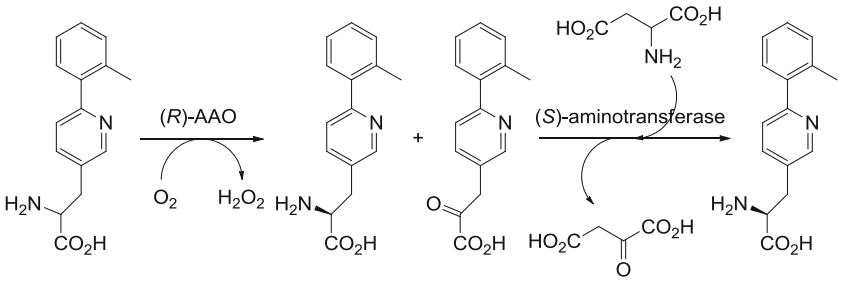

Fig. 7 Enzymatic synthesis of (*S*)-amino-3-[3-{6-(2-methylphenyl)}pyridyl]-propionic acid by cyclic deracemisation using two biocatalysts

properties (Schrittwieser et al. 2011b). Therefore, reticuline oxidase has been thoroughly studied for its use in the synthesis of such alkaloids. The flavoenzyme has been shown to be a potent biocatalyst for performing oxidative coupling reactions to form a range of benzylisoquinolines derivatives with high selectivity. Initial experiments showed that catalase was required in the reaction medium, as the formation of hydrogen peroxide during the oxidative process led to inactivation of the oxidase. Nevertheless, the enzyme is able to maintain its activity in a broad range of pH values (8–11) and temperatures (30–60 °C). As most of the reticuline oxidase substrates are poorly soluble in water, the effect of different organic solvents and ionic liquids on the biocatalyst properties was studied. This revealed that the oxidase is highly tolerant towards solvents. It enabled working at solvent concentrations of 70% *v*/*v* and substrate concentrations of 20 g/L (Resch et al. 2011). Thus, a set of racemic 1-substituted tetrahydroisoquinolines was synthesised starting from the corresponding *N*-phenylmethylamines and phenylacetic acid derivatives and subjected to reticuline oxidase-catalysed resolutions (Schrittwieser et al. 2011a). These oxidase-mediated conversions yielded optically pure products products. Oxidation of (*S*)-reticuline catalyzed by wild-type reticuline oxidase occurs with total regioselectivity, leading to the formation of the 9-hydroxy functionalised regioisomer (*S*)-scoulerine, while the 11-hydroxy regioisomer (*S*)-coreximine was not formed (Fig. 8). The reticuline oxidase E417Q mutant was able to produce a 30% of the 11-hydroxy regioisomer, at the expense of a huge decrease in the reaction rate for the major regioisomer, due to the substrate hydroxyl group depronation and phenyl moiety activation induced by the residue Glu417. As some of the 11-hydroxy functionalised products present biological activity, such as (*S*)-isocoreximine, (*S*)-corytenchine and phellodendrine, reticuline oxidase and its mutant were employed in the oxidative coupling of reticuline analogues, but only traces of the non-expected regioisomers were found. Only substrate modification, by introducing fluoro substituents, led to the synthesis of the 11-hydroxy compounds as sole products with good yields and excellent selectivities (Resch et al. 2012).

Oxidases have also attractive features for use in biosensors. A well-known example of a flavoprotein oxidase that has been used for biosensor development is glucose oxidase (Cash and Clark 2010), which has been extensively applied to monitor the blood glucose levels in diabetics due to its catalytic ability to oxidise glucose. The generated electrons can be used in a direct or indirect manner for readout of a sensor. Another example of an oxidase that can be used for diagnostic purposes is fructosyl amino acid oxidase (EC 1.5.3.x) which oxidises the C–N bond of an amino acid–carbohydrate adduct, resulting in formation of the respective glucosone and amino acid. The oxidase is of interest for detecting glycated proteins, a marker for hyperglycemia in diabetes patients (Qian et al. 2013). Furthermore, D-amino acid oxidases are widely used for the quantification of D-amino acids in biological samples by virtue of their strict stereospecificity (Netto et al. 2013).

Concluding remarks

Oxidases are widespread among a large number of structural flavoprotein families. This shows that nature has found many ways to exploit the ability of the reduced flavin

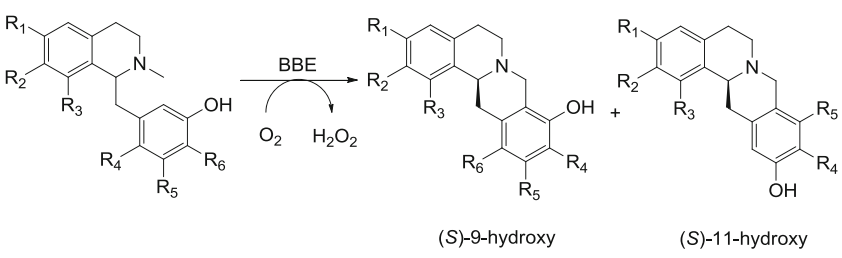

Fig. 8 Reticuline oxidase-catalysed oxidative C–C coupling leading to the 9-hydroxy (major) or 11-hydroxy (minor) stereoisomer

cofactor to use molecular oxygen as oxidant. Interestingly, depending on the structural fold, certain oxidation reactions are preferred. For example, for GMC-type oxidases, there is a strong bias towards alcohol oxidation while members of the sulfhydryl oxidase (SO) family exclusively act on thiols. Yet, oxidases from different structural families can have similar activities. A notable example is cholesterol oxidase for which two structurally distinct representatives have been discovered, one belonging to the VAO oxidase family and the other is a GMC-type oxidase. Another interesting case of shared substrate specificity among two flavoprotein oxidase families has been found for 6-hydroxynicotine oxidases. For each enantiomer of this compound, a specific and structurally distinct oxidase has been found: (*S*)- and (*R*)-6-hydroxynicotine oxidase (respectivelly EC 1.5.3.5 and EC 1.5.3.6). These enzymes oxidise the pyrrolidine ring in the substrate to form the enamine 6-hydroxy-*N*-methylmyosmine. Hydration leads to the final product (*R*)-6-hydroxy-pseudooxynicotine (Decker and Dai 1967). Interestingly, the other enantiomer (*S*)-6-hydroxynicotine is oxidised by an AO-type oxidase.

The flavoprotein oxidases that encompass the broadest range of oxidation activities are the VAO-type oxidases. Reactions of these oxidases include alcohol oxidations, amine oxidation, oxidative ether-bond cleavage, hydroxylations and oxidative C–C and C–O bond formation (Winter and Fraaije 2012). Moreover, the recently discovered VAO-type oxidases that harbour a bicovalently bound FAD have shown to be able act on relatively large substrates. This makes the VAO-type oxidases an interesting oxidase family when looking for new oxidative biocatalysts. With the available sequence and structure data, it has become feasible to discover new oxidases by genome mining. In the last decade, we have been successful in obtaining a number of novel flavoprotein oxidases by this approach: alditol oxidase (Heuts et al. 2007b), eugenol oxidase (Jin et al. 2007), putrescine oxidase (Van Hellemond et al. 2008), and chitooligosaccharide oxidase (Heuts et al. 2007). Genome mining has also shown to be effective in identifying thermostable variants of known oxidases, as shown in L-aspartate oxidase (Bifulco et al. 2013) or alditol oxidase (Winter et al. 2012). This shows that it is becoming relatively easy to identify and produce new oxidases for biotechnological applications. For fine tuning or improving the enzyme properties, ample methods are available to engineer oxidases. It has been demonstrated that glucose oxidase can be optimised for biosensor and biofuel cells, whereas glycine oxidase has been engineered towards converting glyphosate. We have shown that, by enzyme engineering, it is feasible to introduce peroxidase activity into an oxidase (Winter and Fraaije 2012) and to turn a dehydrogenase into an oxidase (Leferink et al. 2009). These examples show that the number of available flavoprotein oxidases is rapidly expanding, which will fuel the development of oxidase-based biotechnological applications.

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